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Empowering malaria vaccination by drug administration

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Although significant progress has been made in clinical development, a protective malaria vaccine remains elusive. Here we review some of the immune subversive mechanisms used by the Plasmodium malaria parasite and propose a potentially effective strategy to achieve complete protection that may serve as a blue print for clinical usage. The premise is to modulate the immune response with drugs that neutralize suppressive functions and potentiate protective responses. Chloroquine may be a first attractive candidate facilitating protective cellular immune responses by improving cross-presentation and reducing suppressive regulatory T cell responses.

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Introduction

Both natural and experimental exposure to malaria parasites can lead to development of protective immunity, providing a strong foothold for the development of a vaccine [1••,2–4,5••]. The development of a malaria vaccine has been a continuous effort over the past half century [6]. Several strategies are being followed, aiming at the sporozoite, liver, blood and/or sexual transmission stages [Figure 1]. The main efforts have been concentrated on development of a sporozoite/liver-stage vaccine against *Plasmodium falciparum*, on the basis of the observation that potent protection could be achieved by inoculation with irradiated non-replicating but metabolically active sporozoites [4].

The field of malaria vaccinology has followed the traditional vaccine approach: expose the host to a malaria antigen or antigens and, in the case of subunit vaccines, maximally stimulate the immune response using adjuvants, immune-stimulatory compounds, or self-adjuvanting delivery systems such as viral vectors or virus-like particles. No vaccine based on this paradigm has worked well, however, for inducing high grade protection against malaria in humans, and the majority of candidates have failed.

Different formulations of a number of antigens have been tested in Phase 1 trials and only about a dozen candidates have been evaluated in Phase 2 clinical field trials (WHO. Date Accessed 19th April 2010. Malaria Vaccine Rainbow Tables; URL: http://www.who.int/vaccine_research/links/Rainbow/en/index.html). The best vaccine to date, RTS,S, does not provide long term protection against infection but delays patency and reduces clinical severity. After several field trials demonstrating roughly 50% protection as measured by delays in the time to acquiring parasitemia or clinical malaria, RTS,S is now undergoing testing in a Phase 3 multicenter trial in Africa [7,8]. Although a milestone in itself and potentially a welcome tool in the combat against malaria, it is clear that better vaccine efficacies will be required in particular for the purpose of malaria elimination [9]. One rational approach would be to counteract the immune-modulating effects of the parasite that result in slow or partial induction of protection and effective memory responses. Co-administration of drugs with immune-modulating properties may be a strategy to meet this objective.

Natural acquisition and evasion of malaria immunity

Malaria parasites generate strong immune responses, and a degree of protective immunity can be acquired through natural exposure, although the mechanisms of protection are poorly understood [1••,2]. The development of this immunity is marked initially by the ability to control the clinical symptoms associated with parasitemia, allowing the individual to tolerate significant parasite densities without overt disease.

The type of clinical immunity, typically developing in children, is followed by resistance to parasitemia, such that older children and adults no longer experience high densities of asexual forms in the blood. However, sterile immunity is never observed in naturally exposed populations; adults living in endemic areas often harbor parasites albeit at low densities and will promptly re-acquire infections if cured through the administration of antimalarial drugs.

Where and how invading *Plasmodia* are recognized and processed by the immune system is not well understood,

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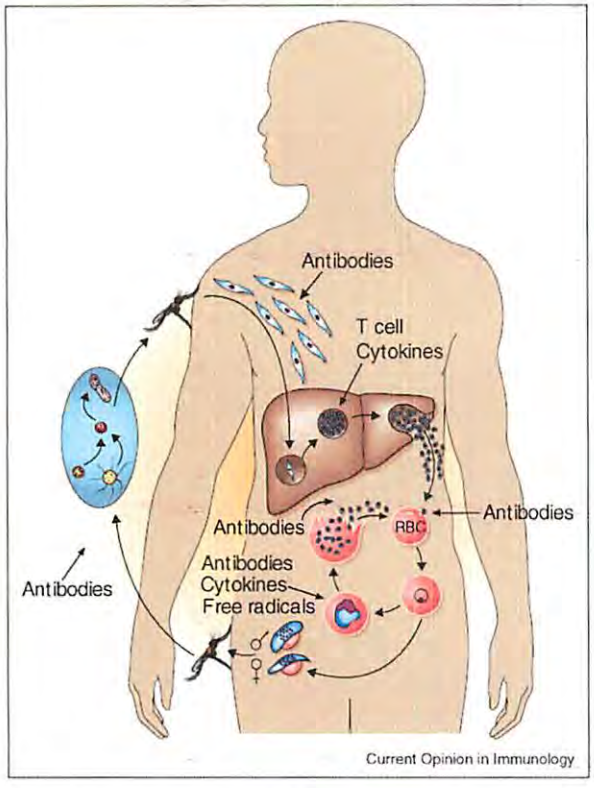
Figure 1



Plasmodium lifecycle. *Plasmodium falciparum* malaria is caused by a protozoan parasite that has a complex multi-stage lifecycle involving both intracellular and extracellular stages in human host and mosquito vector. Malaria infection starts by the bite of an infected *Anopheles* mosquito that inoculates sporozoite forms. Via the bloodstream they reach the liver and invade, mature, and multiply in hepatocytes. Once released by infected hepatocytes into the bloodstream as pathogenic asexual forms they start to multiply in invaded red blood cells. A small fraction of blood stage parasites are committed to become sexual forms and mature into gametocytes that are responsible for transmission to mosquitoes. Mature gametocytes are ingested by blood-feeding mosquitoes and differentiate after a number of transitions into sporozoites and migrate to the salivary gland. At each mosquito bloodmeal these motile parasites are injected into the human host resulting in the spread of the parasite and associated disease in the population. Unlike sporozoites and gametocytes that are clinically silent, only asexual stages of the life cycle are responsible for clinical symptoms, complications, and the possibility of death. Figure is reproduced with permission from Richie TL and Saul A: Progress and challenges for malaria vaccines. *Nature* 2002, 415(6872): 694–701 (Nature Publishing Group).

but probably involves dendritic cells [DCs] with extracellular and intracellular pattern recognition receptors [PRRs]. PRR signal transduction determines the nature of the DC response, and is modulated by the PRRs involved, antigen dose, duration of exposure, and microenvironment. Both TLR-dependent [TLR2, TLR4, and TLR9] as well TLR-independent [NALP3 inflammasome] pathways are involved in *Plasmodium* recognition but major differences between human and murine immune systems hamper conclusive interpretation [10–13]. Activation of DCs preferentially leads to their maturation followed by induction of T effector cells or it may lead to tolerogenic responses by induction of regulatory T cells [Tregs]. The ratio of effector and regulatory responses may influence the risk of productive malaria infection and clinical disease [14]. Protection is accomplished by stage-specific host effector responses and seems to require both cellular and humoral components. While specific antibodies are primarily important against sporozoites and blood stages, distinct cellular responses are required for protection against liver-stage parasites [2]. There is substantial evidence that cytotoxic lymphocytes recognize intra-hepatic parasites and that interferon-γ [IFN-γ] plays an essential role in protection. However, cellular mechanisms are also induced by infected red blood cells [iRBCs] and may control blood stages [3,15].

Thus, malaria manifests itself initially as an acute infectious disease in susceptible persons but evolves into a chronic infection with acquisition of partial immunity. The human immune system gradually controls acute clinical disease, but *Plasmodia* are adapted to prevent complete elimination, establishing a lowgrade, chronic infection in the majority of hosts. This clinical immunity is difficult to acquire and wanes once exposure is withheld. Thus frequent parasite exposure is required to ensure continuity of protection. The efficiency of mounting a protective response is challenged by the morphologically and antigenically distinct lifecycle stages, the existence of genetic and antigenic diversity as well as the parasite's ability to modulate immune responses to its own survival benefit [1••,16••].

Malaria parasites can manipulate recognition by DCs and can compromise the induction of effective immune responses by stimulating Tregs or by other immunosuppressive or immunodiversionary tactics [14,16••,17]. However, the picture in human and animal studies is inconsistent, and the outcome depends on the DC subset as well as the model of parasites and animals studied. While DC maturation may be inhibited or stimulated, the capacity to activate human T cells is generally impaired. Results depend on the subset of DCs studied and the ratio of iRBC to DCs; low ratios are stimulatory while high ratios inhibit or result in apoptosis [16••,17]. Reduced DC function may also be the result of immune inhibition across parasite lifecycle stages as shown in a murine model where asexual stages in the circulation inhibit the generation of effective CD8+ T cell responses targeting liver stages [18]. The DCs exhibit poor maturation and a shifted cytokine profile from primary IL-12 to IL-10 production upon blood stage exposure. In the *P. berghei* model, reduced CD8+ T cell priming is caused by impairment of the cross-presentation by DCs normally essential for antigen presentation by the exogenous route [19]. Also

in humans, inhibition of pre-erythrocytic T cell responses by blood stages is suggested by *in vitro* studies comparing lymphocytes from Duffy antigen-positive individuals [exposed to *P. vivax* blood stages] and Duffy-negative individuals [no exposure to blood stages] [20]. These data fit the epidemiological observation that naturally acquired immunity fails to prevent re-infection even in areas with high infection rates. CD8+ responses are relatively low in individuals from such areas and these effector mechanisms appear incapable of eliminating parasites from the population [21,22]. Thus, DCs that are influenced by blood stages might affect clearance of liver stages upon re-infection.

CD4+CD25+Foxp3+ Tregs are crucial for maintenance of self-tolerance and control of damaging pro-inflammatory responses induced by pathogens including malaria parasites. Expansion of Treg-populations induced by *Plasmodia* is generally seen in both humans and animal models but the functional consequences remain equivocal [14,16••]. Outcomes of functional studies show large variation and, similar to studies of DCs, relate to the model and circumstances chosen. Human Treg activation, however, generally points towards lower pro-inflammatory responses and facilitation of blood stage infection [14]. The balance between Th1 effector cell and Treg responses may determine the clinical presentation. Strong inflammatory responses may overwhelm suppressive Tregs and leave effector T cells unabated with possibly severe clinical symptomatology in an effort to control parasitemia. On the contrary, strong Tregs responses dampen inflammation and symptoms but permit parasitemia and suppress the magnitude of T memory responses possibly owing to competition for IL-2 [14].

Better than nature

Chronic exposure to malaria blood stage parasites may help to explain the slow generation of an effective immune response under natural conditions. Support for this concept comes from rodent studies, where sterile protection against malaria can be achieved by the inoculation of intact sporozoites while treating the animals concomitantly with chloroquine, a drug that kills parasites in the asexual blood stage but not in the pre-erythrocytic liver stage [23]. A proof-of-concept clinical study with a similar protocol in volunteers who had not been previously exposed to malaria likewise showed sterile protection against an experimental *P. falciparum* malaria infection [5••]. This approach exposed volunteers' immune systems to the full course of intra-hepatic development combined with a very brief blood stage parasitemia abrogated by chloroquine. The high degree of protection achieved by a relatively miniscule dose, that is a total of 45 infectious mosquito bites over a period of 3 months, is remarkable. Such inoculation rates may approach the levels in areas of periodical intense transmission in Africa, but sterile protection is not seen under natural conditions. A possible explanation for this difference is the very brief and extremely low parasite density achieved in the blood owing to the killing effect of chloroquine. Parasitemia in nonimmune Africans will, at the minimum, increase to the threshold of clinical symptoms that is at least 1000-fold higher compromising any protection that may result from exposure to sporozoites and liver-stage parasites.

Previously it has been shown that radiation-attenuated sporozoites induce >90% protection in humans [4]. Irradiation of infectious mosquitoes disrupts the gene expression of sporozoites, which remain capable of hepatocyte invasion but are no longer capable of complete liver-stage maturation or progression to the pathogenic blood stage. However, this generally requires 1000 bites by irradiated mosquitoes during five or more immunization sessions, which is a strikingly 20-fold lower potency than the 45 bites in the human model using chloroquine. Similar results are found in mouse studies [23].

Many hypotheses could be generated why intact sporozoites provide substantially better protection than radiation-attenuated sporozoites. These include [i] improved homing to the liver or other sites such as lymph nodes where antigen presentation occurs, [ii] larger antigen yield per sporozoite due to unrestricted asexual reproduction in the liver, [iii] expression of late liver-stage/early blood stage antigens, which are not expressed by irradiated sporozoites. Although these hypotheses are plausible and should be tested, they beg the question why natural exposure to a few mosquito bites does not provide equivalent protection to the experimental model under chloroquine. What other mechanisms might be operative?

Low parasitemia may induce protective effector mechanisms as shown by Pombo *et al.* where repeated intravenous injections of low numbers of parasites followed by a curative treatment before patency resulted in protection against a subsequent blood stage challenge administered without curative drugs [3]. More recently, it was shown in a murine model that subpatent blood stage infection with genetically attenuated blood stage parasites likewise provides complete protection apparently through both humoral and cellular immune responses [24]. By contrast, high parasitemia as observed in the field may inhibit the development of protective immunity and may relate to inhibition of cross-presentation required for the induction of cytotoxic T cells [18–20].

A cost-effective vaccine requires efficient induction of protective immunity over a short period of time and should therefore perform better than nature.

A fresh perspective for failing vaccines

Malaria vaccine development faces a variety of scientific challenges and some of these are addressed by ongoing

subunit vaccine initiatives [8,25,26]. The most advanced among several recombinant protein-adjuvant combinations is RTS,S, a virus-like particle displaying recombinant circumsporozoite protein [CSP] on its surface, expressed together with recombinant hepatitis B surface antigen. The vaccine is formulated with the proprietary adjuvant ASO2A later replaced by its liposomal form ASO1B. Several clinical trials have been conducted in endemic populations including young children age 1–4 years with follow-up up to 45 months. Both anti-CSP antibodies and CD4+ T cell responses show correlation with the observed partial protection. It has proven difficult, however, to achieve significant improvements through combination with other antigens [27].

More recently, vaccine platforms designed to induce cytotoxic T lymphocyte responses targeting hepatic stage parasites through gene-based approaches have achieved partial protection in human volunteers challenged by infected mosquito bites. For example, in a heterologous vaccination strategy, approximately 25% protection was observed both after priming volunteers with a chimpanzee-derived adenovirus vector encoding the TRAP antigen then boosting with a modified vaccinia virus Ankara [MVA] vector also encoding TRAP (AVS Hill, oral presentation, 5th MIM Pan-African Malaria Conference, Nairobi, Kenya; November 2009), and after priming with naked DNA and boosting with a human-derived adenovirus vector [serotype 5] with both platforms encoding CSP and AMA1 (I. Chuang *et al.* poster presentation, given at Malaria: New Approaches to Understanding Host-Parasite Interactions, Keystone Symposia on Molecular and Cellular Biology, Copper Mountain, Colorado; April 2010). Whether the modest protection achieved by viral vectors can be improved by the addition of antigens is not yet known.

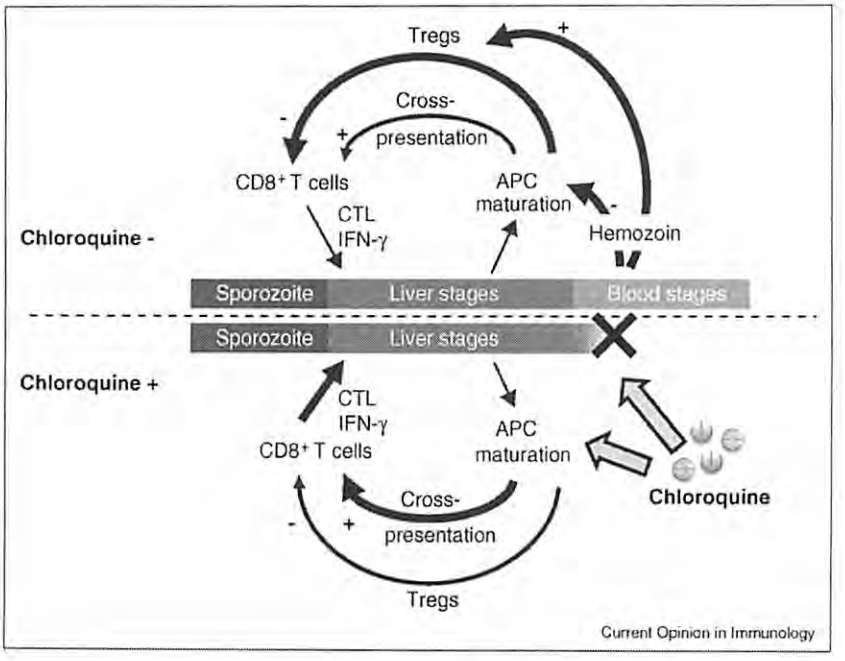
One approach to enhance the protection afforded by subunit vaccines is to combine recombinant protein in adjuvant with viral vectors. The former induces primarily antibodies and helper T cell responses, while the latter induce strong cell mediated immunity including cytotoxic CD8+ T cells. Combined into heterologous regimens, these two approaches might powerfully activate both the humoral and cellular arms of the immune system, thereby enabling the destruction of multiple parasite stages [28]. However, all subunit vaccines must overcome the challenge of antigenic heterogeneity and the difficulty of protecting a genetically heterogeneous population.

Intact parasites attenuated by irradiation [4], genetic modification [29], or by co-administration of antimalarial drugs [3,5••,23], show a superior degree of protection to subunit vaccines in animal studies and in controlled experimental human infections. Presenting potentially hundreds of antigens to the immune system, these approaches are designed to provide 'something for everyone' thereby circumventing the limitations of genetic restriction by broadening the antigenic repertoire. A radiation-attenuated sporozoite vaccine is currently undergoing clinical testing, while a genetically attenuated sporozoite vaccine will soon follow into the clinic. Although it is too early to judge the success of these approaches, some may be confronted with the generation of immune suppressive mechanisms normally used by wild type parasites thereby limiting their ability to protect. A major challenge may be to redress such malaria-associated suppressive mechanisms.

Co-administration of chloroquine may enhance protection induced by sporozoite inoculation owing to lowering of parasitemia and/or its immune-modulating effects. Short-course treatment of mice with chloroquine improves the priming of naïve CD8+ T cell responses against soluble antigens *in vivo* [30]. Cross-presentation of soluble viral antigens to specific CD8+ T cell clones *in vitro* is improved when DCs are pulsed with the antigen in the presence of chloroquine. Furthermore, if hepatitis B virus vaccine [HBV] responders are further boosted together with a single dose of chloroquine a substantial increase of HBV-specific CD8+ T cells is observed [31]. Presentation of soluble viral antigens to specific CD8+ T cell clones by DCs is greatly improved in the presence of chloroquine, which prevents endosomal acidification, and seems to promote the transfer of endocytosed material into the cytosol. The net effect will depend on the routing and processing conditions such as acidification of the endosomal compartment [32•].

There may be more drug choices to explore for co-administration during vaccination. Chemotherapeutic drugs have shown potential benefits for the immune response against tumors [33,34]. It has for instance been shown that low dose cyclophosphamide selectively depletes Tregs in both animal models and cancer patients with resulting enhancement of T cell functions. The potential benefits and safety risks of low dose cytostatic drugs in healthy volunteers should be carefully considered. The combined data show that orchestration of the antigen presenting pathways by drug modulation might tailor the immune response to a desired profile. The functionality of the increased numbers of Tregs observed in human and animal malaria is not clear but there is an inverse correlation during acute disease between Tregs and malaria-specific memory responses [35•]. Antigen presentation by immature or partially mature DCs conditions the emergence of Tregs. To activate Tregs, TLR9 signaling in dendritic cells is required and mediated by hemozoin, a digestion product of hemoglobin produced by *Plasmodium* that is involved in TLR9 activation [11]. Since chloroquine abrogates hemozoin-mediated cytokine production, this drug might inhibit this evading mechanism leading to a more effective establishment of immunological memory [36] [Figure 2].

Figure 2



Model for modulation of cellular responses by chloroquine during malaria. Blood stage parasites inhibit the development of liver-stage immunity via antigen presenting cells and hemozoin. This results in upregulation of Tregs and therefore less effective establishment of CD8+ T cell responses. Chloroquine prevents the development of blood stage parasitemia, thereby diminishing the immune-evasing action of these parasites. Furthermore, it inhibits Treg induction and improves cross-presentation, therefore leading to a more effective CD8+ T cell response.

Does combining a parenterally administered vaccine with orally administered chloroquine or other immunomodulatory drug hold any practical application as a novel vaccination strategy? Given the long half-life of chloroquine, a single administration might maintain effective plasma levels throughout the induction phase of an immune response, making it possible to administer single doses of co-drug and vaccine as the immunization procedure, although the effectiveness of this reduced-dose approach would need to be tested. In the RUNMC study, the vaccine component consisted of intact *P. falciparum* sporozoites, presenting a potentially insurmountable safety concern [5••]. However, both radiation-attenuated and genetically attenuated sporozoite approaches should demonstrate very low levels of breakthrough blood stage infection, if they occur at all, and thus could serve as ideal partners for an immunomodulatory co-drug. A first step would be to test these combinations in order to determine the minimal protective dose of both vaccine and co-drug, as well as the fewest number of immunizations needed to achieve high grade protection. If chloroquine or another antimalarial were chosen, this approach could provide an added level of safety since it would treat any emergent blood stage infections even if the sporozoite vaccine were only partially attenuated. As long as the vaccine strain was completely sensitive to the co-drug, both immune modulation and protection against breakthrough blood stage infections could be achieved at the same time. Practical application will depend on the selected drug, drug half-life, and vaccination regime.

Conclusion

In malaria where immune diversionary mechanisms are a primary immune-evasion strategy, co-administration of immune-modulating drugs along with the vaccine may be required in order to achieve high grade protection. The hypothesis of vaccination with drug co-administration assumes that the vaccine component mimics the natural pathogen in terms of subverting the host immune responses. This is a reasonable assumption in the case of whole organism vaccines such as attenuated sporozoites.

This approach might be less applicable to a subunit vaccine based on discrete single antigens, although even in this instance there may be immunodominant immune responses [repeat motifs, for example, that, if suppressed, would allow more protective, subdominant responses to emerge]. The current armamentarium of immunomodulatory agents used to affect the immune system including cytostatic drugs could have potential as co-agents and be screened for activity. Clearly dose and duration of treatment will be important, not too high and not too low, to strike the balance to interfere with the

pathogen's adaptation strategy without preventing the host immune system from responding adequately and safely.

Chloroquine may be the first opportunity to test and this could be done by comparing the administration of chloroquine with that of less immune-modulating antimalarials, or by giving chloroquine with irradiated or genetically attenuated sporozoites to see if the potency of the vaccine is increased. Despite more than 30 years of chloroquine resistance in Africa, this antimalarial drug is still widely available and used for presumed treatment. Although responsible for increasing numbers of treatment failures, one may hypothesize that the immune-modulating effects of chloroquine may still have some contribution to development of clinical protection.

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